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New insights into the Manila clam – *Perkinsus olseni* interaction based on gene expression analysis of clam hemocytes and parasite trophozoites through *in vitro* challenges

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Abstract

The Manila clam (*Ruditapes philippinarum*) is the bivalve species with the highest world production from both fisheries and aquaculture, but its production is seriously threatened by perkinsosis, a disease caused by the protozoan parasite *Perkinsus olseni*. To understand the molecular mechanisms underlying *R. philippinarum–P. olseni* interaction, we analyzed the gene expression profiles of *in vitro* challenged clam hemocytes and *P. olseni* trophozoites, using two oligo-microarray platforms, one previously validated for *R. philippinarum* hemocytes and a new one developed and validated in this study for *P. olseni*. Manila clam hemocytes were *in vitro* challenged with trophozoites, zoospores, and extracellular products from *P. olseni in vitro* cultures, while *P. olseni* trophozoites were *in vitro* challenged with Manila clam plasma along the same time-series (1 h, 8 h, and 24 h). The hemocytes showed a fast activation of the innate immune response, particularly associated with hemocyte recruitment, in the three types of challenges. Nevertheless, different immune-related pathways were activated in response to the different parasite stages, suggesting specific recognition mechanisms. Furthermore, the analyses provided useful complementary data to previous *in vivo* challenges, and confirmed the potential of some proposed biomarkers. The combined analysis of gene expression in host and parasite identified several processes in both the clam and *P. olseni*, such as redox and glucose metabolism, protease activity, apoptosis and iron metabolism, whose modulation suggests cross-talk between parasite and host. This information might be critical to determine the outcome of the infection, thus highlighting potential therapeutic targets. Altogether, the results of this study aid to understand the response and interaction between *R. philippinarum–P. olseni* and will contribute for developing effective control strategies for this threatening parasitosis.

Keywords: Manila clam, perkinsosis, *in vitro* challenge, gene expression, hemocytes, host-parasite interaction

1. Introduction

The Manila clam *Ruditapes philippinarum* (Veneridae; Adams and Reeve, 1850) is the bivalve mollusk with the highest world production and a major contributor to the livelihoods of many coastal communities in Europe and Asia. However, the sustainability of Manila clam production is threatened worldwide by perkinsosis, a disease caused by the parasite *Perkinsus olseni*. Over the years, perkinsosis mass mortality events have been recorded in Manila clam both in Asia (Liang et al., 2001; Wu et al., 2011; Nam et al., 2018; Waki et al., 2018) and Europe (Sanmartí et al., 1995; Pretto et al., 2014). Despite perkinsosis seriously concerns clam farmers, no treatment or effective preventive measures are currently available for this disease.

The life cycle of *P. olseni* includes four developmental stages, namely zoospore, trophozoite, prezoosporangium (hypnospore) and zoosporangium, all involved in the direct transmission of perkinsosis from host to host (Villalba et al., 2004). Zoospore is a biflagellated free swimming stage that transforms into trophozoite once it has invaded the host (Wang et al., 2018). Trophozoite is the most observed stage in the host, where it vegetatively proliferates; trophozoites released from the host, either through diapedesis or in the feces, are able to infect other clams (Villalba et al. 2004); furthermore, trophozoites give rise to prezoosporangia in moribund hosts (Villalba et al., 2004; Casas and La Peyre, 2013). In seawater, prezoosporangia develop into zoosporangia, where hundreds of zoospores are produced and eventually released (Casas et al., 2002a).

Several studies have been carried out to understand the response of Manila clam to *P. olseni* and the lesions associated with the disease (reviewed in Soudant et al., 2013); particularly profuse are those highlighting the role of the hemocytes, multifunctional cells of the hemolymph which infiltrate the infected tissue. Recently, the genetic response of Manila clam hemocytes to perkinsosis has been characterized through high-throughput transcriptomic sequencing (Hasanuzzaman et al., 2017) and microarray analyses either after *in vivo* challenge with trophozoites (Romero et al., 2015) or zoospores, as well as wild exposure (Hasanuzzaman et al.,

2018). Furthermore, the protein profiles of clam hemocytes and plasma after *in vivo* challenge with zoospores have also been reported (Fernández-Boo et al., 2016).

Clam perkinsosis has also been studied from the parasite point perspective focusing on different biological processes, including its proliferation (Casas et al., 2002b; Elandalloussi et al., 2003, 2005a; Araujo et al., 2013), metabolic pathways (Elandalloussi et al., 2005b), pathogenicity (Shimokawa et al., 2010; Waki et al., 2012; Waki and Yoshinaga, 2013), population genetic structure (Pardo et al., 2011; Vilas et al., 2011), and gene and protein expression profiles (Ascenso et al., 2007; Leite et al., 2008; Ascenso, 2011; Fernández-Boo et al., 2014, 2015a, 2015b). Moreover, the transcriptome of *P. olseni* trophozoites challenged with Manila clam plasma has been recently assembled and annotated (Hasanuzzaman et al., 2016). As described for the congener species *Perkinsus marinus* (Villalba et al., 2004; Pales Espinosa et al., 2014), the extracellular products released by *P. olseni* appear to be involved in the pathogenesis of the disease (Fernández-Boo et al., 2014, 2015b). However, to our knowledge, the differential response of hemocytes to the different *P. olseni* life stages and its extra-cellular products has not been investigated, and no studies have provided an integrated view of the host-parasite interaction. Host resistance and parasite virulence are intricately intertwined, and thus their responses should be studied in parallel to thoroughly understand the host-parasite interaction (Nelson, 1973; Day, 1974).

High throughput technologies, including microarrays and RNA-Seq, are commonly used to study the genetic responses underlying host-parasite interactions (Coyne et al., 2011; Hughes et al., 2011; Manque et al., 2011; Leite et al., 2013). Microarrays have become a universal tool for analyzing the expression of thousands of genes (Bubendorf, 2001; Allison et al., 2006; Sobek et al., 2006) and have been used in the past to understand different biological processes in Manila clam (Milan et al., 2011; Moreira et al., 2012, 2014; Allam et al., 2014; Menike et al., 2014; Romero et al., 2015). Functional information might be combined with other studies addressed to identify molecular markers useful for breeding programs. For example, selection to increase resistance of *Crassostrea virginica* against diseases, including the infection with the congener species *P.*

marinus, have been successfully implemented in the USA (Frank-Lawale et al., 2014; Proestou et al., 2016; Casas et al., 2017).

Here, we developed and validated the first microarray platform for *P. olseni* trophozoites, which was applied, along with the previously developed for *R. philippinarum* hemocytes (Hasanuzzaman et al., 2018), to analyze host-parasite interaction *in vitro* across a time series (1 h, 8 h, and 24 h) under different scenarios: a) *P. olseni* trophozoites challenged with *R. philippinarum* plasma; and b) clam hemocytes challenged with *P. olseni* trophozoites, zoospores and extracellular products. The results obtained complement the information from previous studies of perkinsosis in Manila clam and provide new insights on the processes underlying *R. philippinarum*-*P. olseni* interaction. This knowledge is critical to devise successful disease prevention strategies.

2. Materials and methods

2.1. Experimental design and sampling

R. philippinarum collected from a *P. olseni*-free area (Camariñas, NW Spain) were used to collect hemocytes for the *in vitro* challenge with *P. olseni* trophozoites, zoospores and extracellular products (proteins released into the culture medium by *P. olseni* trophozoites). Similarly, *P. olseni* trophozoites were challenged *in vitro* with *R. philippinarum* plasma collected from clams of the *P. olseni*-free area. The details for collection of hemocytes, trophozoites, zoospores and extracellular products have been described in Hasanuzzaman et al. (2017). The procedures to collect trophozoites from *in vitro* cultures (1-2 months old) and the isolation of Manila clam plasma have been described in Hasanuzzaman et al. (2016). Absence of *P. olseni* infection in every used clam was confirmed by PCR and incubation of gill pieces in Ray's fluid thioglycollate medium. All experiments were carried out in the facilities of Centro de Investigacións Mariñas (CIMA; Spain).

2.1.1. In vitro challenge of clam hemocytes with Perkinsus olseni

137 *R. philippinarum* hemocytes ($5x10^6$) were challenged *in vitro* with *P. olseni* trophozoites
138 ($5x10^6$), zoospores ($5x10^6$) and extracellular products (2.5 mL of culture media enriched with
139 extracellular products) separately in IWAKI 6-well plates (Fig.1A). Each challenge included three
140 biological replicates for both treatment and control (only culture media) groups, and each biological
141 replicate was a pool of hemocytes from 10 different clams, thus averaging individual biological
142 variation. For the challenges, trophozoites and zoospores, obtained just before the challenge, were
143 separately suspended in 2.5 mL filtered seawater (FSW) and added into a permeable insert (0.2 µm
144 Anopore® membrane NUNC 25 mm) in each well. For hemocyte-extracellular products challenge,
145 2.5 mL of culture media enriched with hemocyte extracellular products were added into the inserts
146 of the respective wells. The inserts allowed the flow of media but not the cells; hence, hemocytes
147 and parasite cells were never in contact. Samples for RNA extraction were collected at 1, 8 and 24 h
148 after the start of the challenge. Further details are available in Hasanuzzaman et al. (2017).
149
150 ***2.1.2. In vitro challenge of P. olseni trophozoites with Manila clam plasma***
151 *P. olseni* trophozoites (~ $5x10^6$) resuspended in 2.5 mL FSW were placed in IWAKI 6-well
152 plates, and 2.5 mL of plasma (treatment) or FSW (control) were added into a permeable insert (0.2
153 µm Anopore® membrane NUNC 25 mm) set in the plate-wells (Fig. 1B). Four pseudo-replicates
154 (trophozoites from the same culture) for both control and treatment groups were collected at 1, 8
155 and 24 h since the onset of the challenge. Further details are available in Hasanuzzaman et al.
156 (2016).
157
158 **2.2. RNA extraction**
159 Total RNA was extracted using Qiagen RNeasy mini kit with DNase treatment following
160 manufacturer's instructions. The RNA quality and quantity were evaluated in a Bioanalyzer (Bonsai
161 Technologies) and a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies Inc)
162 respectively.

2.3. Microarray analyses

2.3.1. R. philippinarum hemocyte microarray experiment

The design of the *R. philippinarum* oligo-microarray has been described and validated in Hasanuzzaman et al. (2018). Briefly, this microarray was designed on an 8 × 15 k Agilent format and included 14,621 probes representing 11,052 transcripts of which 10,813 were annotated (97.8%).

Thirty-two microarrays (four slides) were used. *R. philippinarum* control replicates at each time point were pooled, so a single control microarray was used for each sampling time (1-C, 8-C, 24-C); in addition, the low RNA amount in the controls of the zoospore challenge determined pooling all controls in a single microarray hybridization.

2.3.2. P. olseni trophozoite microarray design and experiment

To construct the *P. olseni* oligo-microarray, we selected 10,104 sequences from our previously published *P. olseni* trophozoite transcriptome (Hasanuzzaman et al., 2016). A total of 9,369 sequences were selected because they were annotated to transcripts in the NCBI nr protein database, while the remaining 735 non-annotated sequences were selected by their notable differential expression in our preliminary evaluation (Hasanuzzaman et al., 2016). One oligo-probe was designed for annotated sequences (known sense) and two probes (sense and antisense) were designed for the non-annotated sequences. We also included 4,158 technical replicates for microarray reproducibility evaluation. All processes for oligo-probe design and Agilent oligo-microarray procurement were similar to those followed for the Manila clam microarray (Hasanuzzaman et al., 2018).

A total of 16 microarrays (two slides) were used for the experiment. A single pooled control was hybridized in two microarrays per slide as technical replicates. Twelve microarrays were used for the treatments across the time-series (1 h, 8 h and 24 h) including four replicates per time point.

2.3.3. Hybridizations and analysis

Hybridizations were performed at the Universidade de Santiago de Compostela (USC) Functional Genomics Platform using the Agilent Technology Gene Expression Unit following a one-color gene expression analysis protocol. All hybridizations were carried out by the same researcher in the same day. Hybridized slides were scanned using an Agilent scanner (G2565B, Agilent Technologies) and signals were captured and processed. The microrarray platforms (Agilent-072098 and Agilent-xxx) and data presented in this publication has been deposited in the NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are available under accession number xxxx. Microarray hybridization, data processing and quality filtering were carried out as previously described (Hasanuzzaman et al., 2018). Briefly, the gProcessedSignal value was used for statistical analyses, and the spatial detrend correction was applied using the Agilent Feature Extraction algorithm. Normalization within each microarray was carried out applying the loess method. After data quality evaluation, only probes with absolute fluorescence values > 200 were considered robust for further microarray analyses. We determined the variation and reproducibility associated with both biological and technical replicates by estimating Pearson correlation coefficients. Further, Spearman´s Rho correlation between identical probes for the same gene was estimated across all microarrays.

Hierarchical Clustering, Principal Component Analysis (PCA), Self Organizing Tree clustering Algorithm (SOTA) and other statistical analyses, such as *t*-test and ANOVA were performed using MultiExperiment Viewer version 4.9.0 (tMev) of the TM4 Microarray Software Suite (Saeed et al., 2006). Differentially expressed genes (DEGs) between treatment and control samples were detected with a t-test using a false discovery rate (FDR) of 0.05 (SAM analysis). DEGs between different challenges and times were detected using a two-way (for *R. philippinarum* microarray) and one-way (*P. olseni*) ANOVA. DEGs were functionally characterized with Gene Ontology (GO) terms using Blast2GO Version 3.2 (Conesa et al., 2005) with default parameters.

Enriched GO terms were determined by comparing the set of DEGs in a comparison with the full set of annotated transcripts of either Manila clam or *P. olseni* using Blast2GO Fisher's exact test (FDR = 0.05).

2.4. Microarray validation

Quantitative PCR (qPCR) was performed to validate the results of both *R. philippinarum* hemocyte and *P. olseni* trophozoite microarrays. Some samples had not enough RNA for qPCR after microarray hybridization, and therefore 22 out of 25 and 8 out of 12 microarrays were validated for *R. philippinarum* and *P. olseni*, respectively. Moreover, since the remaining *P. olseni* control RNA quantity was not enough for qPCR analysis, we used as control one of the treatment replicates of 1h that showed a highly significant positive correlation (r = 0.85) with control microarrays and further, it was positioned very close to controls in the PCA analysis and far away from the two other 1 h treatment biological replicates. A significant and high positive correlation was detected between qPCR and microarray data in the *P. olseni* trophozoites challenge (see Results) supporting our strategy.

To select genes for validation, we followed the random stratified procedure proposed by Miron et al. (2006) with some modifications as described by Millán et al. (2011). Log2 fold change (FC) variation of each probe across all experimental conditions was determined and the standard deviation (SD) estimated. Taking into account the SD range and gene annotation (preferably immune-related genes), we selected 14 and 7 genes for qPCR validation in the Manila clam and *P. olseni* experiments, respectively. Thereafter, FCs of these genes were ordered in ascending values for the 22 *R. philippinarum* microarrays (14 genes × 22 microarrays = 308 cases) and the 8 *P. olseni* microarrays (7 genes × 8 microarrays = 56 cases), and then subdivided into a series of strata. A total of 39 cases were selected in the *R. philippinarum* experiment considering strata FC range, and at least one gene per microarray and one case per gene. For *P. olseni*, 19 cases were selected considering FC range, with at least three genes per microarray and one case per gene. Two

241 reference genes were selected considering their least expression variation across microarrays and
242 literature suggestions (Filby and Tyler, 2007; Infante et al., 2008): 60S ribosomal protein L18
243 (*RPL18*) and 40S ribosomal protein S12 (*RPS12*) for Manila clam, and 60S ribosomal protein L12
244 and 40S ribosomal protein S3 for *P. olseni* qPCR validation.

245 Primers were designed using *Primer Express* Software v2.0 (Applied Biosystems) with
246 default settings (Table S1). Primer specificity to the PCR template (i.e. selected gene) was checked
247 usingNCBI/Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The qPCR analysis
248 was conducted as described by Hasanuzzaman et al. (2018).

249

250 ## 3. Results

251 ### 3.1. Quality of microarray data

252 The *R. philippinarum* hemocyte microarray fluorescence values exhibited a very high
253 reproducibility between technical replicates within microarrays ($r > 0.99$, $p < 0.001$). Correlation
254 between biological replicates was also high ($r > 0.88$, $p < 0.01$). Hierarchical clustering and PCA
255 analyses showed appropriate clustering of biological replicates at each time point in all experiments.
256 After removing those probes with absolute fluorescence values < 200, the number of remaining
257 probes per microarray ranged from 4,295 to 5,961.

258 Similarly, the *P. olseni* microarray showed high reproducibility ($r > 0.98$, $p < 0.01$; Pearson
259 correlations for absolute fluorescence values between replicates). The hierarchical clustering and
260 PCA analyses showed sample clustering according to the time point series, except for one of the
261 replicates at 1 h, which as previously mentioned (See Material and Methods section 2.4) clustered
262 close to controls. After removing probes with absolute fluorescence values < 200, the number of
263 remaining probes was 6,500, 6,493 and 6,679 for 1, 8 and 24 h respectively.

264

265 ### 3.2. Validation of microarray data by quantitative real-time PCR

Twelve genes showed a significant correlation between *R. philippinarum* hemocyte microarray and qPCR data ($r = 0.68$; $p < 0.01$), and there were no significant differences between both datasets ($t = 0.860$; $p = 0.397$). The ubiquitin B and serine protease genes both demonstrated late amplification and dissociation curves with multiple peaks when analyzed by qPCR and therefore were excluded from further analyses.

The expression of selected *P. olseni* genes by qPCR and microarray was also significantly correlated ($r = 0.76$; $p < 0.01$) and no significant differences were found between both datasets ($t = 1.570$; $p = 0.134$).

The significant correlation of microarrays and qPCR for the selected genes supports the consistency of our microarray platforms. The minor discrepancies might be explained by the lower RNA quality of some samples, the putative existence of chimaeras in our transcriptome databases derived from the bioinformatics assembling pipeline, and the existence of paralogous genes and/or splice variants; genomic expansion of gene families has been observed in bivalves, such as the expansion of stress-related genes in Pacific oyster *Crassostrea gigas* (Zhang et al. 2012). Resulting paralogous genes could complicate the interpretation of microarray results due to cross-hybridization.

3.3. Gene expression profiles in *R. philippinarum* hemocytes

3.3.1. Differential expression between control and P. olseni-challenged hemocytes

The number of DEGs varied greatly across the time-series and in response to the different *P. olseni* stages (Fig. 2; Table S2). Further, the response of hemocytes to the different challenges was rather specific with few common up- or down-DEGs (Fig. 3). The highest number of DEGs were shared between extracellular products and trophozoite challenges at 24 h, while nearly 50% DEGs (up- and down-regulated) were shared between extracellular products (8 h) and trophozoites (1h) datasets (Fig. S1). On the other hand, the response to zoospores was very specific; the highest

number of DEGs was detected at 1 h, mostly up-regulated, while nearly only down-regulated genes were detected at 8 h (207 of 208 DEGs) and only 4 DEGs were found at 24h.

The top DEGs (FC > |3|) for the three challenges (Tables 1, 2 and 3) were mostly time-specific. DEGs encoding different structural components of ribosomes, NADH dehydrogenase subunit 4 (*ND4*) and ubiquitin isoform cra_e were detected in the three types of challenge. Some stress- and immune-related genes, such as those from the family of cytochrome p450 (e.g. *CYP2H2*-like, *CYP18A1*, *CYP2J2*-like), complement c1q-like protein 4 (*C1QL4*), serum amyloid a (*SAA*) and ras-related c3 botulinum toxin substrate 2 (*RAC2*) were found among the top DEGs in the hemocytes challenged with trophozoites and zoospores; however, their modulation was less intense but still significant in the challenge with the extracellular products (Table S2). Other genes, such as tandem repeat galectin (*TRGal*) or apolipoprotein d (*APOD*), were also significantly modulated in all the scenarios (Table S2), although only appeared as top DEGs in one of the challenges.

The GO-term analyses rendered several enriched functions presumably related to the Manila clam defense response in the three challenges (Fig. S2). GO terms related to the activation of innate immune response, particularly hemocyte recruitment (vascular endothelial growth factor receptor signaling pathway, positive regulation of macrophage chemotaxis), were found in the three challenges at 1h. Endopeptidase activity, redox processes, iron metabolism, protein synthesis, cytoskeleton structure and mitosis/cell proliferation were found enriched in more than one challenge.

Other recurrent GO terms showed a particular pattern regarding the challenge and/or the time; GO-terms related to the toll-like receptors and MAPK signaling pathways were up-regulated in response to the zoospores at 1 h, but down-regulated in the challenges with the extracellular products at 8h and the trophozoites at 24 h. A similar pattern was also observed for NIK/NFkB and RAS transductional signaling, both up-regulated in hemocytes challenged with zoospores at 1 h, but down-regulated in response to extracellular products at 8 h. Moreover, the GO term negative regulation of apoptotic processes was down-regulated at 8 and 24 h in response to extracellular

317 products and trophozoites, respectively, while monooxygenase activity was up-regulated in both
318 cases either at 8 h or 24 h.

319 The analysis also identified some enriched functions that appeared challenge-specific; the
320 up-regulation of genes involved in cell junction assembly and platelet aggregation in response to
321 extracellular products, the activation of natural killer cell mediated cytotoxicity processes in the
322 case of the challenge with trophozoites, or that of the TNF signaling in response to zoospores.

323

324 ***3.3.2. Differences between hemocytes challenged with different P. olseni stages***

325 A two-way ANOVA analysis detected 2,996 DEGs between challenges (trophozoites,
326 zoospores or extracellular products; Fig.4, Tables S3, S4, S5) and 2,014 DEGs between times (1 h,
327 8 h, 24 h; Fig. 5; Tables S6, S7, S8). There was a strong differential response of hemocytes to the
328 different *P. olseni* stages or extracellular products (Table S3). A self Organizing Tree Algorithm
329 (SOTA) analysis identified a total of nine clusters of DEGs showing highly correlated expression
330 profiles across challenges ($r > 0.9$) (Table S4, Fig. S3A). GO-term enrichment was analyzed within
331 each SOTA cluster (Table S5). In group P-SG4 (Fig. 4), GO terms related to redox processes and
332 endopeptidase inhibitory activity were enriched, and genes appeared to be down-regulated in
333 response to extracellular products at 8 h, while up-regulated in response to trophozoites at 24 h. In
334 group P-SG8, genes related to cysteine-type peptidase activity were found again up-regulated in the
335 trophozoite challenge at 24 h, while down-regulated in response to zoospores (Fig. 4). Indeed, the
336 enriched GO terms of groups P-SG8 and P-SG9 revealed that, starting from 8 h, the hemocytes
337 challenged with zoospores showed a notable down-regulation of genes related to the immune
338 response (chemokine activity and chemotaxis processes), defense response (apoptosis signaling
339 pathway, mitosis), as well as cellular structure (actin filament and actomyosin structure
340 organization) and metabolism (glycolysis and gluconeogenesis, ATP synthesis, lipid binding) (Fig.
341 4). Moreover, GO terms related to protein synthesis, extracellular matrix (laminin receptor, focal
342 adhesion), stress (ubiquitin activity) and phosphatidylinositol-mediated signaling were enriched in

group P-SG6, characterized by genes up-regulated in the extracellular products challenge at 24 h, but slightly down-regulated in response to trophozoites and zoospores (Fig. 4).

We also analyzed the expression profile of the 50 more discriminating genes between challenges to look for particular and combined patterns that could aid to characterize the different parasite stages across the time-course (Fig. S4). These genes precisely distinguished the hemocyte response by challenge and time since all replicates were clustered within the same group. Extracellular products challenge was grouped in a single cluster close to trophozoite challenge, while zoospore challenge was clustered in a differentiated group related to trophozoite 8 h. The clustering of these 50 genes showed four main gene profiles separated by horizontal bars in Figure S4. Regrettably, a certain amount of these genes could not be consistently annotated, which limits their utility, but several stress response and apoptosis-related genes were identified characterizing stages / times across infection. A future analysis and annotation of these genes will aid to understand the response to the different parasite stages involved in the infection process.

3.3.3. Time-specific gene expression in IVT challenged hemocytes

The 2,014 DEGs showing significant differences across times (two-way ANOVA; Fig.5; Table S6) were split into nine subgroups of genes with highly correlated patterns ($r > 0.9$) using the SOTA algorithm (Table S7, Figure S5). Two of these groups (T-SG2, T-SG3) showed quite similar expression patterns in hemocytes in response to extracellular products and trophozoites, while a different pattern was observed in response to zoospores (Fig. 5, Table S7). These genes, up-regulated in response to extracellular products and trophozoites at 8 h, were enriched in specific GO terms: heme binding, related to iron metabolism, and respiratory chain (group T-SG2, Table S8, Fig. 5A), or GO-terms related to immune response, apoptosis and cytoskeleton (group T-SG3; Table S8; Fig. 5B). Gene expression for these two groups showed an opposite pattern in the

zoospore experiment at 1 h, being down-regulated in T-SG2 and up-regulated in T-SG3, while, by contrast, their expression was reduced at both 8 and 24 h.

3.4. Gene expression in *P. olseni* trophozoites challenged with Manila clam plasma

The FC values of *P. olseni* trophozoites challenged with Manila clam plasma ranged between -2.4 and +3.9 (Table S9). A total of 65 DEGs were detected at 1 h (42 up- and 23 down-regulated), 48 at 8 h (37 up- and 11 down-regulated), and 300 at 24 h (201 up- and 99 down-regulated) (Fig. 6). At 24 h most DEGs (46%) were either not annotated or annotated as hypothetical proteins (Table S9). GO-term annotation revealed that most up-regulated genes at 1 h were associated with stress-response, iron-sulfur cluster assembly and cell-redox homeostasis, while most down-regulated genes were related to proteolysis. Oxidation-reduction processes and proteolysis appeared again among the overrepresented functions at 8h and 24 h. At 8 h, there was a clear trend showing the genes associated to oxidation-reduction processes among those up-regulated, while proteolysis-related genes were down-regulated. By contrast, most proteolysis-related genes were up-regulated at 24 h, as well as several genes associated with glycolysis, directly involved in cell redox homeostasis (e.g. gamma-enolase, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), fructose-1,6-bisphosphate aldolase (*FBA*)).

A one-way ANOVA ($p < 0.05$ with standard Bonferroni correction) identified a total of 886 DEGs along the time series (Table S10). The SOTA clustering algorithm identified two groups of genes (Table S11, Fig. S5). The first group (258 genes) included genes up-regulated at 1 h and down-regulated at 24 h, while the second (628 genes) presented an opposite pattern. This last group showed a significant enrichment in multiple GO terms related to DNA replication (Table S12), likely associated with imminent cell proliferation.

4. Discussion

In this study, we analyzed the interaction between Manila clam and *P. olseni* through an *in vitro* time course experiment using hemocytes and plasma of the host, and different life stages and extracellular products of the parasite. This study complements our previous *in vivo* challenge (Hasanuzzaman et al., 2018) and aids to dissect the genetic interaction between the parasite and the hemocytes at the initial stages of infection.

Several genes, such as *SAA*, *ND4*, *CYP450*, cathepsins and *APOD*, suggested as potential biomarkers in the *in vivo* challenge (Hasanuzzaman et al., 2018) were also found differentially expressed in all the scenarios here analyzed, most of them among the top DEGs in one or more challenges. This confirms their key role during perkinsosis and their potential for further investigations aimed to achieve perkinsosis-resistant strains in selective breeding programs. These genes are involved in processes previously identified as relevant in the pathogenesis of perkinsosis, such as innate immunity (*SAA*), cell redox homeostasis and reactive oxygen species (ROS) production (*ND4* and *CYP450*), apoptosis and lysosomal degradation processes (cathepsins) and lipid metabolism (*APOD*) (Leite et al., 2013; Soudant et al., 2013; Romero et al., 2015; Hasanuzzaman et al., 2018). *SAA* is among the most powerful acute-phase proteins in vertebrates, being involved in immune-cell chemotaxis and cytokine secretion (Badolato et al., 1995; Ribeiro et al., 2003), and these functions as mediators of the inflammatory response appear to be conserved in bivalves (Rosani et al., 2016). Cell migration and infiltration of hemocytes in parasitized tissues mediated by chemo-attractant stimuli had been previously reported during perkinsosis infected clams (Villalba et al., 2004; Soudant et al., 2013). In Manila clam, the recruited hemocytes are responsible for the encapsulation of the parasite (Montes et al., 1995a, 1995b), which would then be attacked and destroyed by the synergistic effects of ROS and lysosomal enzymes (Soudant et al., 2013). Another gene involved in ROS production whose expression was strongly modulated in the different challenges was *RAC2*. This gene encodes a small Rho GTPase necessary for NADPH oxidase full assembly, but it is also involved in actin cytoskeleton organization occurring in chemotaxis and phagocytosis (Tell et al., 2012). The down-regulation of *RAC2* in response to

zoospores and trophozoites at different times deserves further work to investigate its role during perkinsosis, considering the multiple evidences indicating that *Perkinsus* spp. is able to interfere with the phagocytic capacity of hemocytes as well as its certain tolerance to ROS (Soudant et al., 2013). Another protein related to Rho GTPases, namely Rho GTPase activating protein 6, had been pinpointed as a possible marker for resistance to *P. olseni* (Fernández-Boo et al., 2016).

Our study also highlights the role of lectins and C1q domain containing proteins in perkinsosis, both involved in innate immunity through pathogen cell recognition and agglutination processes (Kishore et al., 2004; Kim et al., 2008a, 2008b); particularly, the C1q-like lectin *C1QL4* and *TRGal* were differentially expressed in the three challenges. The activation of lectin complement pathway via C1q-like lectins seems to be a key mechanism in the innate immunity of Manila clam in response to *P. olseni* infection (Prado-Alvarez et al. 2009; Leite et al., 2013, Hasanuzzaman et al., 2018). Likewise, cytoskeleton organization was found modulated in response to the different challenges, mostly through up-regulation of genes at early stages (1 h) in response to zoospores and through down-regulation at later times, when in turn their expression increased in the challenges with trophozoites and extracellular products. This process plays an essential role in the response of immune-related cells such as hemocytes (May and Machesky, 2001), but host cytoskeleton is also a common and recurring target for the infectious strategies of pathogenic microbes (Gruenheid and Finlay, 2003). Modulated expression of cytoskeleton-related genes was previously reported in perkinsosis-infected Manila clam (Romero et al., 2015; Fernández-Boo et al., 2016; Hasanuzzaman et al., 2017, 2018).

The response of hemocytes to zoospores was the most differentiated one in our study, particularly at 1 h, where the most intense modulation was detected. Comparing the *in vitro* response of hemocytes challenged with zoospores with that previously observed *in vivo* (Hasanuzzaman et al., 2018), we found several common enriched GO terms between 1 and 10 h post challenge. Particularly interesting is the enrichment of MAPK activity, toll-like receptors and NIK/NFκB signaling pathways among up-regulated genes, which in turn appeared among those

444 down-regulated at different time points in the challenges with trophozoites and extracellular
445 products in the present study.

446 Mitogen-activated protein kinases (MAPK) signal cascades, characterized as the most
447 ancient and evolutionarily conserved signaling pathways (Widmann et al., 1999), are involved in
448 cell growth, apoptosis, inflammation and response to environmental stresses. One of the well-
449 studied downstream components of the MAPK signaling pathway is the nuclear transcription factor
450 kappa B (NFκB), directly involved in regulating different immune functions (Akanda and Park,
451 2017; Sun, 2017). As well, toll-like receptors (TLRs) act as primary sensors that detect a wide
452 variety of microbial components by the recognition of pathogen-associated molecular patterns
453 (Allam and Raftos, 2015), and TLR signaling pathway plays pivotal roles in host innate immune
454 defense mechanism. The downstream signaling after pathogen detection by TLRs includes the
455 activation of MAPK and NFκB, which regulate the expression of cytokines, chemokines and
456 interferons that ultimately protect the host from microbial infection (Kawasaki and Kawai, 2014).
457 This kind of response was only found in the hemocytes challenged with zoospores in our study and,
458 together with the expression of cytoskeleton-related genes, suggests a different mechanism of
459 recognition between zoospores and trophozoites that would activate different immune-related
460 pathways in the host. Later on, a broad down-regulation was found in the zoospore challenge at 8 h,
461 involving genes related to a variety of relevant cell functions, while almost no DEGs were found at
462 24 h, reflecting a return to a homeostasis state. Further work is needed to elucidate if this pattern
463 could be induced by *P. olseni* as a strategy associated with its transformation from zoospore into
464 trophozoite, which appears to occur soon after host invasion, but the mechanisms involved are not
465 clear yet (Wang et al., 2018).

466 As expected, we found more similarities between the hemocyte response to trophozoites and
467 their extracellular products. Particularly, the response against extracellular products at 8 h was
468 clustered with the response against trophozoites at 1 h (~ 50% shared DEGs), thus it appears to be
469 delayed, although the analyses indicated that both responses start to converge at 24 h. The most

relevant mechanisms that were similarly regulated in these challenges were redox and glucose metabolism, protease activity, apoptosis and iron metabolism. The integrated analysis of the changes in the transcriptome of both the hemocytes and the trophozoites of *P. olseni* allowed us to gain more insight into these functions involved in Manila clam - *P. olseni* interaction.

In host-parasite interactions, metabolic-related genes are usually significantly regulated; the parasite requires energy for its survival, growth and reproduction, while the host needs energy to elicit the immune response and cope with the infection. These bioenergetic needs of cells are met by the interconnected pathways of glycolysis, tricarboxylic acid cycle and oxidative phosphorylation (Ganeshan and Chawla, 2014). Particularly, up-regulation of glycolysis is a critical step in the activation of immune cells, to synthesize macromolecules and generate the antimicrobial respiratory burst (Ganeshan and Chawla, 2014). ROS production is an important microbicidal mechanism of hemocytes, but, if it is not well counter balanced by their cellular antioxidant capacity, leads to oxidative stress, which is often associated with the pathogenesis of infectious diseases (Ray et al., 2012). We found that pathways related to oxidation-antioxidation were regulated in both the parasite and the host, particularly active in hemocytes challenged with trophozoites at 24 h, confirming a critical role in their interaction, as suggested in our previous studies (Hasanuzzaman et al., 2016, 2017, 2018). Evidence of oxidative stress and activation of antioxidant defenses by hemocytes included the modulation of *APOD*, which might have a role as an antioxidant molecule during perkinsosis (Hasanuzzaman et al., 2018), but also *TXNDC*, acting in thioredoxin system, or glutathione S-transferases, *GST*s. Up-regulation of antioxidant-related genes was also detected in *P. olseni* trophozoite, such as *AHPC*, a member of family peroxidases involved in controlling endogenous and exogenous peroxides in response to stress (Hofmann et al., 2002), or *GST*, peroxiredoxin 5 and membrane selenoprotein, previously related to antioxidant activity in *Perkinsus* spp. (Araujo et al., 2013, Fernández-Boo et al., 2014, 2015a, 2015b). On the other hand, the down-regulation in the trophozoite of prostatic acid phosphatase precursor might hinder the ability of *P. olseni* to deal with ROS, since this gene has been reported to play a role in the eradication of

hydrogen peroxide (H_2O_2) and the inhibition of O_2^- in *P. marinus* (Volety and Chu, 1997; Soudant et al., 2013; Pales Espinosa et al., 2014).

Interestingly, some DEGs that act in redox and glucose metabolism detected in both the host and the parasite, such as enolase, *GAPDH* and *FBA* are also known to provide function as plasminogen receptors (Yang et al., 2010; Ghosh and Jacobs-Lorena, 2011; González-Miguel et al., 2013), and might be involved in enhancing the virulence of the parasite. Pathogens are capable of modulating host cells to acquire and / or recruit plasminogen / plasmin, which can degrade immunoglobulin, complement molecules and extracellular matrix proteins (Jolodar et al., 2003; Chung et al., 2011), thus facilitating adherence, evasion of the immune response, tissue penetration and migration, and nutrition uptake (Kitt and Leigh, 1997; Jolodar et al., 2003; Yavlovich and Rottem, 2007; Siemens et al., 2011; Figuera et al., 2013). Particularly, *GAPDH*, has been shown to inhibit complement C3 activity, modulating the immune response of the host of the parasite *Haemonchus contortus* (Sahoo et al., 2013). In this study, the expression of different genes related to plasminogen metabolism was modulated in both the trophozoites and hemocytes, particularly in the hemocytes challenged with extracellular products. In that challenge we found down-regulation of various complement components at 8 h and 24 h, and enriched GO terms related to extracellular matrix at 24 h (DEGs found by the ANOVA analysis between challenges). This could be also associated with the enrichment of the platelet aggregation signaling pathway (directly related with plasminogen / plasmin) found in the hemocytes against extracellular products at 1 h and 8 h. Platelets play a prominent role in tissue repair and regeneration in vertebrates, with relevant connection with several pathways of immune response and apoptosis (Gawaz and Vogel, 2013). In invertebrates, hemocytes serve as multipurpose defense cells, which include a role in the processes of tissue repair and remodeling (Cerenius and Söderhäll, 2010). The early up-regulation of the platelet aggregation pathway could then be a response induced by the tissue damaging factors contained in extracellular products, responsible for the characteristic tissue degradation observed in perkinsosis (Pales Espinosa et al., 2014; Fernández-Boo et al., 2015b). A potential challenge-

specific response can be found in the down-regulation of genes related to phosphatidylinositol biosynthesis and protein glycosylation, also detected at 24 h, possibly aimed at the inhibition of trophozoite proliferation by reducing glycosylated-phosphatidylinositol anchors, which demonstrated to be essential for survival in several parasite species (Martin and Smith, 2006; Ferguson et al., 2017).

On the other hand, the enrichment of the GO term "natural cell mediated cytotoxicity processes" was the most characteristic result in the hemocytes challenged with trophozoites at 1 h. This suggests the triggering of a cytotoxic response against that parasite stage by the hemocytes, that were previously reported to present this natural killer-like activity in different invertebrates (Franceschi et al., 1991; Parrinello, 1996; Chernysh et al., 2004). The different onset of the hemocyte response against the trophozoite and extracellular products might underlie the different timing observed in the regulation of redox and glucose metabolism, as well as apoptosis and proteases activity.

Cathepsins-related genes, whose expression appeared widely regulated in this study, are responsible for driving proteolytic degradation within the lysosome and in the extralysosomal milieu, and under certain conditions, such as ROS production, they are released in the cytoplasm and participate in the execution of apoptosis (Chwieralski et al., 2006; Repnik et al., 2012). This has been considered a relevant host defense mechanism against the infection by *Perkinsus* spp (Soudant et al., 2013; Romero et al., 2015), and in this study we found several evidences of apoptosis modulation. Particularly, the expression of cathepsins and apoptosis-related genes was mostly up-regulated in the hemocytes challenged with trophozoites at 1 and 24 h and in those challenged with extracellular products at 8 and 24h. Nevertheless, we found some evidences that *P. olseni* might interfere with the apoptotic machinery as a strategy to facilitate its proliferation by suppressing host-cell apoptosis at certain stages of the infection, as reported for other parasitosis (Keller et al., 2006; Sokolova, 2009; Gervais et al., 2018). In fact, some apoptotic genes were inhibited in hemocytes after being exposed to *P. olseni* stages, particularly in the challenge with zoospores

548 starting from 8h, after their initial up-regulation at 1 h. Also, the up-regulation of Bax inhibitor in *P.*
549 *olseni* trophozoite and the down-regulation of *BCL2* in the hemocytes challenged with trophozoites
550 at the same time point (24 h) might represent a direct interaction of Bax inhibitor with *BCL2* to
551 modulate apoptosis in host cells (Xu and Reed, 1998).

552 The expression of several genes encoding cathepsins and other proteolysis-related genes was
553 also modulated in *P. olseni* trophozoites, mostly down-regulated at early stages and up-regulated at
554 24 h, when the parasite showed the most intense transcriptomic changes. It is well known the
555 importance of proteases as virulence factors, involved in the invasion, immune evasion, nutrition,
556 and reproduction of parasites (Armstrong, 2006; Lilburn et al., 2011). This has been also porved for
557 *Perkinsus* spp. in a study related to *P. marinus* virulence (Pales Espinosa et al., 2014) and by the
558 presence of proteolytic factors amongst *P. olseni* extracellular products (Fernández-Boo et al.,
559 2015b). On the other hand, the host response involves the production of protease inhibitors that are
560 capable of inactivating and clearing the proteases involved in parasitic invasion (Armstrong, 2006).
561 These inhibitors have demonstrated to play a key role for resistance in some cases, such as the
562 serine protease inhibitor cvS-1 of oysters against *P. marinus* (La Peyre et al., 2010). This issue still
563 remains to be elucidated in Manila clam-*P. olseni* interaction; in our work, we did not find a clear
564 increase in the expression of protease inhibitors in challenged hemocytes, although a potential
565 candidate could be alpha macroglobulin (up-regulated in the challenge with *P. olseni* trophozoites),
566 which is an important component of the invertebrate innate immune response capable of binding
567 and neutralizing the diverse array of proteases that function as virulence factors (Armstrong, 2010).

568 Finally, it is well-known the relevance of iron metabolism during protozoa infections, where
569 a constant battle between the host and the invader around this element, essential for parasite
570 survival, has been reported (Weinberg, 2009). Iron availability was demonstrated to be involved in
571 the proliferation, virulence and metabolic pathways of *P. olseni*, being iron metabolism proposed as
572 a promising therapeutic target (Elandalloussi et al., 2003, 2005a; Leite et al., 2008; Araujo et al.,
573 2013). In this work, several genes involved in iron metabolism were regulated in the hemocytes,

again with a similar expression pattern in challenges against trophozoites and extracellular products (up-regulated), while their down-regulation was observed in response to zoospores at 1 h post-challenge. The most relevant DEGs were those encoding ferritins, the main iron-storage proteins, whose increased gene expression indicates an iron withholding defense system (Weinberg, 2009), and proteins involved in the biogenesis and assembly of iron-sulfur (FeS) clusters, such as *ISCA1* (Cózar-Castellano et al., 2004). FeS clusters are among the most ancient and versatile protein cofactors, playing both structural and catalytic roles, acting in central metabolic processes such as electron transfer, redox chemistry, enzyme catalysis, and sensing environmental or intracellular conditions to regulate gene expression (Beinert et al., 1997; Dellibovi-Ragheb et al., 2013). In a previous study on the transcriptome of *P. olseni* stimulated with clam plasma, Hasanuzzaman et al. (2016) reported the modulation of genes encoding FeS assembly protein, and here we also found in challenged trophozoites at 1 h several up-regulated genes involved in this pathway. These included the iron-sulfur cluster assembly protein *SUFB*, a member of the sulfur mobilization system, a pathway of the FeS biogenesis machinery that is present in plastid-containing organisms, such as the apicomplexan parasites, where it is secluded to the apicoplast organelle (Dellibovi-Ragheb et al., 2013). There are multiple evidences indicating that *Perkinsus* spp., closely related to Apicomplexa, contains a plastid organelle (Joseph et al., 2010). In particular, *de novo* isoprenoid synthesis, a characteristic process occurring in that organelle, was detected in both *P. marinus* and *P. olseni* (Matsuzaki et al., 2008; Hasanuzzaman et al., 2016). The SUF pathway in the plastid would be responsible for supplying FeS clusters to proteins involved in isoprenoid synthesis, a process strongly suggested to be essential for the viability of parasites, making it an attractive candidate for the development of new drug targets (Dellibovi-Ragheb et al., 2013).

5. Conclusions

The comparison of the different Manila clam - *P. olseni* cell/product challenges across the time course has provided new useful data to understand the host-parasite interaction during

perkinsosis. Globally, the analyses revealed a fast response of the hemocytes to *P. olseni*, modulating a substantial number of genes involved in innate immune responses, but *P. olseni* zoospores, trophozoites and extracellular products showed to trigger differential transcriptomic changes in hemocytes, especially connected with the early activation of different immune-related pathways. Our results aid to understand the hemocyte response to the different parasite stages, including their interaction with parasite virulence factors throughout the infection process. Moreover, the relevance of several genes previously indicated as potential biomarkers for perkinsosis was confirmed here, and the integrated analysis of host and parasite transcriptomic changes highlighted the main mechanisms involved in this host-parasite interaction, providing new candidate molecules and therapeutic targets to control perkinsosis in Manila clam.

Acknowledgements

This work was funded by the Ministerio de Economía y Competitividad of the Spanish Government, through the projects (AGL2011-30449-C02-01 and AGL2012-37981), the European Regional Development Funds (FEDER 2007-2013) and the Regional Government of Galicia, Xunta de Galicia, through the projects ED431C 2018/28 and ED431D 2017/21. The study was also supported by the Centro de Supercomputación de Galicia (CESGA). The first author would like to acknowledge the PhD scholarship awarded by the EXPERTS III Consortium of the European Community Mobility Programme "Erasmus Mundus Action 2, Strand 1" (EMA2). SFB was supported by a scholarship of the Consellería do Mar da Xunta de Galicia. The authors are also grateful to Lucía Insua, Maribel Meléndez and Elena Penas for providing technical assistance.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://data.mendeley.com/datasets/zvnw69chcz/draft?a=b53e943a-aec2-4646-ad4b-3f3646b13c13

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931

Legends to Figures

Figure 1. Experimental design of *in vitro* challenges. (A) Manila clam hemocytes (H) *vs* *Perkinsus olseni* trophozoites (T), zoospores (Z) and extracellular products (ECP) from *in vitro* cultured parasites; (B) *P. olseni* T *vs* Manila clam plasma. FSW= filtered seawater. RFTM = Ray's fluid thioglycollate medium.

Figure 2. Number of differentially expressed genes in Manila clam hemocytes (H) challenged with *Perkinsus olseni* extracellular products (ECP), trophozoites (T) and zoospores (Z) along a time series. Up- and down-regulated genes are shown above and below the horizontal axis, respectively, and their intensity with a colour degree from light to dark according with their FC.

Figure 3. Venn diagrams showing the number of down- and up-regulated genes across the time course in Manila clam hemocytes (H) challenged with *Perkinsus olseni* extracellular products (ECP), trophozoites (T) and zoospores (Z).

Figure 4. Most relevant functionally enriched SOTA groups from Manila clam hemocytes (H) differentially expressed genes (Parasite Factor; ANOVA, $p < 0.001$) after challenging with different *Perkinsus olseni* parasite stages/products (ECP = extracellular products, T = trophozoites and Z = zoospores) between challenge types. A) P-SG4; B) P-SG6; C) P-SG8; D) P-SG9.

Figure 5. Most relevant functionally enriched SOTA groups from Manila clam hemocytes (H) differentially expressed genes (Time Factor; ANOVA, $p < 0.001$) after challenging with different *Perkinsus olseni* parasite stages/products (ECP = extracellular products, T = trophozoites and Z = zoospores) across a time series. A) T-SG2; B) T-SG3.

Figure 6. Differentially expressed genes in the *in vitro* stimulated *Perkinsus olseni* trophozoites (P-T) challenged with Manila clam plasma through a time series. Up- and down-regulated genes are shown above and below the horizontal axis, respectively, and their intensity with a colour degree from light to dark according with their FC.

957

958 **Legends to Supplementary Files**

959

960 **Figure S1.** Venn diagrams comparing differentially expressed genes of *Perkinsus olseni* hemocytes
961 against trophozoite (H-T) at 1h post challenge *vs*. extracellular products (ECP) at 8h post challenge;
962 A) down-regulated genes; B) up-regulated genes; C) all genes.

963 **Figure S2.** Gene Ontology (GO) term enrichment of up- and down-regulated genes in *R.*
964 *phillipinarum* hemocytes after *in vitro* challenges with *Perkinsus olseni* extracellular products
965 (ECP), trophozoites (T) and zoospores (Z) at 1 h, 8 h and 24 h post-challenge.

966 **Figure S3.** Nine SOTA groups including highly correlated gene profiles identified among the
967 differentially expressed genes of *Perkinsus olseni* hemocytes between challenges using a two-way
968 ANOVA**. B**. Nine SOTA groups including highly correlated gene profiles identified among the
969 differentially expressed genes across the time series using a two-way ANOVA.

970 **Figure S4.** Hierarchical clustering of differentially expressed genes in Manila clam hemocytes
971 (Parasite Factor, ANOVA, $p < 0.001$) after challenging with different *Perkinsus olseni* parasite
972 stages/products (ECP = extracellular products, T = trophozoites and Z = zoospores).

973 **Figure S5**. SOTA groups including highly correlated gene expression profiles among the
974 differentially expressed genes of *Perkinsus olseni* trophozoites after a challenge with *R.*
975 *phillipinarum* plasma using a one-way ANOVA.

976 **Table S1**. qPCR primers used to validate *Perkinsus olseni* microarray data.

977 **Table S2.**- Differentially expressed genes of *R. phillipinarum* hemocytes (FDR = 5 %; FC > 1
978 or < -1) after challenges with several *Perkinsus olseni* forms across a time series (1h, 8h,
979 24h).

Table S3.- Differentially expressed genes ($p < 0.001$; $1 < FC < -1$) between hemocyte *Perkinsus olseni* challenges (extracellular products, ECP; trophozoites, T; and zoospores, Z) across the time series (1 h, 8 h, 24 h) using a two-way ANOVA (factor challenge).

Table S4.-Nine groups of hemocyte differentially expressed genes (factor *Perkinsus olseni* form, ANOVA, $P < 0,001$) showing a significant correlation pattern ($r > 0.90$; SOTA groups).

Table S5.-Enriched GO terms in the nine SOTA groups identified among the differentially expressed genes detected in hemocytes between *Perkinsus olseni* challenges (ANOVA, $p < 0.001$).

Table S6.- Differentially expressed hemocyte genes ($p < 0.001$; $1 < FC < -1$) after *Perkinsus olseni* challenges between times (1 h, 8 h, 24 h) using a two-way ANOVA (factor time).

Table S7.-Differentially expressed genes of hemocytes after *Perkinsus olseni* challenges in the three most relevant SOTA groups (factor time, ANOVA, $P<0,001$) showing a significant correlation pattern (SOTA groups) in the experimental conditions analyzed.

Table S8.-Enriched GO terms in the three most relevant SOTA groups of differentially expressed genes of hemocytes (factor time, ANOVA, $P<0,001$) after *Perkinsus olseni* challenges showing a significant correlation pattern (SOTA groups) in the experimental conditions analyzed.

Table S9.- Differentially expressed genes of *Perkinsus olseni* trophozoites (FDR = 5 %; $FC > 1$ or < -1) after challenge with Manila clam plasma along a time series (1h, 8h, 24h).

Table S10.- Differentially expressed genes of *Perkinsus olseni* trophozoite after a challenge with Manila clam plasma across a time series using a one-way ANOVA ($p < 0,05$ after Bonferroni correction)

Table S11.-Two groups of trophozoites differentially expressed genes (ANOVA, $P < 0,05$ after Bonferroni correction) after challenge with Manila clam plasma showing a significant correlation pattern ($r > 0,90$; SOTA groups).

Table S12.- Enriched GO terms in the two SOTA groups of differentially expressed genes of trophozoites (ANOVA, P<0,05) after challenge with Manila clam plasma showing a significant correlation pattern (SOTA groups) in the experimental conditions analyzed.

1010 **Table 1**. Top differentially expressed genes (FC > |3|) annotated to known proteins in the Manila
1011 clam hemocytes challenged with *Perkinsus olseni* extracellular products.

Annotation	GO terms	Log_2 FC*		
		1 h	*8 h*	*24 h*
NADH dehydrogenase subunit 4	C:mitochondrion; F:NADH dehydrogenase (ubiquinone) activity; P:ATP synthesis coupled electron transport; P:oxidation-reduction process	-	4.6	-
Alpha amylase	F:hydrolase activity, acting on glycosyl bonds; P:carbohydrate metabolic process	-	4.1	-
60s ribosomal protein l26	C:large ribosomal subunit; F:structural constituent of ribosome; P:translation	-	3.9	-
50s ribosomal protein l14	C:large ribosomal subunit; F:structural constituent of ribosome; P:translation	-	-	3.9
Type i inositol -trisphosphate 5-phosphatase 12 related	F:inositol-polyphosphate 5-phosphatase activity; P:protein dephosphorylation	-	3.9	-4.4
Asparagine synthetase	F:asparagine synthase (glutamine-hydrolyzing) activity; P:glutamine metabolic process; P:negative regulation of apoptotic process	-	3.2	-
Protein odd-skipped-related 2 isoform x2	F:nucleic acid binding; P:positive regulation of cell proliferation	-	-	3.1
40s ribosomal protein s15aa	C:ribosome; F:structural constituent of ribosome; P:mitotic spindle elongation	-	3.0	-
Cartilage matrix	C:collagen trimer; F:calcium ion binding	2.5	-3.6	-
Ubiquitin-60s ribosomal protein l40	C:extracellular space; F:structural constituent of ribosome; P:protein polyubiquitination; P:stimulatory C-type lectin receptor signaling pathway	-	-4.2	-4.3
60s ribosomal protein l23a-like	C:ribosome; F:structural constituent of ribosome; P:translation	-	-4.0	-
Inosine-uridine preferring nucleoside hydrolase-like	F:hydrolase activity	-3.9	-	-3.3
Cytosolic phospholipase a2	C:intracellular membrane-bounded organelle; F:phospholipase activity; P:phospholipid catabolic process	-	-3.9	-
Actin isoform zwei	C:focal adhesion; F:structural constituent of	-	-3.7	-

	cytoskeleton; P:small GTPase mediated signal transduction; P:cell junction assembly			
Protein phosphatase 1 regulatory subunit 12a-like	C:intracellular part	-	-3.4	-
Ubiquitin isoform cra_e	C:extracellular space; F:protease binding; P:protein polyubiquitination	-	-3.2	-
40s ribosomal protein s3	C:focal adhesion; F:structural constituent of ribosome; P:translational initiation; P:negative regulation of apoptotic process	-	-3.2	-
Tubulin beta chain isoform x1	C:microtubule;F:structural constituent of cytoskeleton;P:microtubule-based process; P:natural killer cell mediated cytotoxicity	-	-	-3.2

*Fold change (FC) is shown as an average of expression values across replicates. P: Biological Process, C: Cellular Component, F: Molecular Function.

Table 2. Top differentially expressed genes (FC > |3|) annotated to known proteins in the Manila clam hemocytes challenged with *Perkinsus olseni* trophozoites.

Annotation	GO terms	Log_2 FC*		
		1 h	*8 h*	*24 h*
Cytochrome p450 2j2-like	C:intracellular part; F:oxidoreductase activity; P:organic acid metabolic process	-	4.3	-
c-type lysozyme 2	F:lysozyme activity; P:metabolic process	3.9	-	-
Serum amyloid a	C:extracellular space; F:Toll-like receptor 4 binding; P:I-kappaB phosphorylation	3.8	-	-
Cytochrome p450 2h2-like	F:binding; P:metabolic process	-	3.8	-
Cytochrome p450 18a1	C:intracellular membrane-bounded organelle; F:oxidoreductase activity; P:xenobiotic metabolic process	-	3.7	3.1
Early growth response protein 1-b-like	F:nucleic acid binding; F:metal ion binding	-	-	3.7
Apolipoprotein d-like	F:lipid binding; C:extracellular region; P:transport; P:negative regulation of cellular process	-	-	3.6
Ubox domain containing protein	C:ubiquitin ligase complex; P:protein ubiquitination; F:ubiquitin-protein transferase activity	-	-	3.5
NADH dehydrogenase subunit 4	C:mitochondrion; F:NADH dehydrogenase (ubiquinone) activity; P:ATP synthesis coupled electron transport; P:oxidation-reduction process	3.4	-	-
Protein plant cadmium resistance 3-like	-	3.3	-	-
Ubiquitin partial	C:extracellular space; F:protease binding;P:protein polyubiquitination; P:stimulatory C-type lectin receptor signaling pathway	3.2	-	-
Steroid 17-alpha-hydroxylase lyase	F:oxidoreductase activity; F:metal ion binding	-	3.2	-
Cytochrome p450 2j6-like	F:monooxygenase activity;P:oxidation-reduction process	-	3.2	-
Heavy metal-binding protein hip-like	-	3.1	-	-
Complement c1q-like protein 4	F:carbohydrate binding; C:collagen trimer	-	3.1	-
60s ribosomal protein l23a-like	C:ribosome; F:structural constituent of ribosome; P:translation	-	-	-5.5

Alpha skeletal muscle	C:nuclear chromatin; F:protein kinase binding; P:skeletal muscle thin filament assembly	-	-	-5.3
Ubiquitin isoform cra_e	C:extracellular space; F:protease binding; P:protein polyubiquitination	-	-	-5.1
Type i inositol -trisphosphate 5-phosphatase 12 related	F:inositol-polyphosphate 5-phosphatase activity; P:protein dephosphorylation	-	-	-5.1
Actin isoform zwei	C:focal adhesion; F:structural constituent of cytoskeleton; P:small GTPase mediated signal transduction; P:cell junction assembly	-	-	-5.0
Guanine nucleotide-binding protein subunit beta-2-like 1	C:phagocytic cup; F:protein kinase C binding; P:positive regulation of protein phosphorylation; P:activation of cysteine-type endopeptidase activity involved in apoptotic process	-	-	-4.9
40s ribosomal protein s3	C:focal adhesion; F:structural constituent of ribosome; P:translational initiation; P:negative regulation of apoptotic process	-	-	-4.7
Cholesterol 7-alpha-monooxygenase-like	C:endoplasmic reticulum; F:monooxygenase activity; P:steroid metabolic process	-	-	-4.3
Caspase-3-like isoform x2	F:cysteine-type peptidase activity; P:apoptotic process; P:proteolysis	-	-	-3.8
Alpha amylase	F:hydrolase activity, acting on glycosyl bonds; P:carbohydrate metabolic process	-	-	-3.5
Signal transducer and activator of transcription 5b-like	C:intracellular; P:hemopoiesis; P:cell differentiation	-	-	-3.4
Ras-related c3 botulinum toxin substrate 2	C:intracellular part; F:nucleotide binding; P:cellular metabolic process; P:response to stimulus	-	-	-3.3
Interferon regulatory factor 2	F:DNA binding	-	-	-3.1

* Fold change (FC) was shown as an average of expression values across replicates. P: Biological Process, C: Cellular Component, F: Molecular Function.

1019 **Table 3**. Top differentially expressed genes (FC > |3|) annotated to known proteins in the Manila
1020 clam hemocytes challenged with *Perkinsus olseni* zoospores.
1021

Annotation	GO terms	Log_2 FC*		
		1h	*8h*	*24h*
Tandem repeat galectin	C:immunological synapse; F:protein binding; P:leukocyte chemotaxis	6.3	6.5	-
40s ribosomal protein s16-like protein	C:focal adhesion; F:structural constituent of ribosome; P:rRNA processing;	3.7	-	-
Cytochrome p450 2h2-like	F:binding; P:metabolic process	3.7		4.7
Elongation factor 1- partial	C:cytoplasm; F:translation elongation factor activity; P:translational elongation	-	-	4.3
Eukaryotic initiation factor 4a	F:translation initiation factor activity; P:translational initiation	-	-	3.9
Cytochrome p450 18a1	C:intracellular membrane-bounded organelle; F:oxidoreductase activity; P:xenobiotic metabolic process	3.5	-	-
Cytochrome p450 2j2-like	C:intracellular part; F:oxidoreductase activity; P:organic acid metabolic process	3.3	-	-
Alpha skeletal muscle	C:nuclear chromatin; F:protein kinase binding; P:skeletal muscle thin filament assembly	-10.7	-4.4	-
Ubiquitin isoform cra_e	C:extracellular space; F:protease binding; P:protein polyubiquitination	-10.0	-5.2	-
NADH dehydrogenase subunit 4	C:mitochondrion; F:NADH dehydrogenase (ubiquinone) activity; P:ATP synthesis coupled electron transport; P:oxidation-reduction process	-6.9	-8.2	-
60s ribosomal protein l26	C:large ribosomal subunit; F:structural constituent of ribosome; P:translation	-6.5	-7.5	-
ORF16-lacz fusion protein		-	-7.3	-
40s ribosomal protein s3	C:focal adhesion; F:structural constituent of ribosome; P:translational initiation; P:negative regulation of apoptotic process	-6.4	-4.6	-
Ubiquitin-60s ribosomal protein l40	C:extracellular space; F:structural constituent of ribosome; P:protein polyubiquitination; P:stimulatory C-type lectin receptor signalling pathway	-	-5.8	-

40s ribosomal protein s16	C:focal adhesion; F:structural constituent of ribosome; P:rRNA processing	-	-5.6	-
Guanine nucleotide-binding protein subunit beta-2-like 1	C:phagocytic cup; F:protein kinase C binding; P:positive regulation of protein phosphorylation; P:activation of cysteine-type endopeptidase activity involved in apoptotic process	-	-5.5	-
Actin isoform zwei	C:focal adhesion; F:structural constituent of cytoskeleton; P:small GTPase mediated signal transduction; P:cell junction assembly	-	-4.7	-
Heavy metal-binding protein hip-like	-	-4.6	-3.8	-
60s ribosomal protein l23a-like	C:ribosome; F:nucleotide binding; P:translation	-	-4.6	-
Complement c1q-like protein 4	F:carbohydrate binding; C:collagen trimer	-4.1	-	-
CALRL protein	C:endoplasmic reticulum; F:calcium ion binding; P:protein folding	-3.8	-	-
Galaxin	-	-3.7	-	-
Serum amyloid a	C:extracellular space; F:Toll-like receptor 4 binding; P:I-kappaB phosphorylation	-3.7	-	-
Ras-related c3 botulinum toxin substrate 2	C:intracellular part; F:nucleotide binding; P:cellular metabolic process; P:response to stimulus	-3.6	-	-
c-type lectin domain family member a	F:monosaccharide binding	-3.5	-	-
Serum amyloid a-5	C:extracellular space; F:chemoattractant activity; P:cell chemotaxis	-3.4	-	-

* Fold change (FC) was shown as an average of expression values across replicates. P: Biological Process, C: Cellular Component, F: Molecular Function.